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Novel Fucolipids of Human Adenocarcinoma: Characterization of the Major Le^y Antigen of Human Adenocarcinoma as Trifucosylnonaosyl Le^y Glycolipid ($III^3FucV^3FucVI^2FucnLc_6$)*

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A series of glycolipid antigens with Le^y determinant (Fucα1→2Galβ1→4(Fucα1→3)GlcNAc) defined by monoclonal antibody AH6 (Abe, K., McKibbin, J. M., and Hakomori, S. (1983). *J. Biol. Chem.* 258, 11793–11797) have been detected in human colonic carcinoma cases. Three Le^y-active components have been identified as follows. (i) The simplest compound was characterized as Le^y hexaosylceramide (lactodifucohexaosylceramide, $III^3FucIV^2FucnLc_4$), which was previously isolated and was found as the major component in six out of eight cases of colonic adenocarcinoma but as only a very minor component in two cases. (ii) The second component was a very minor component in all eight cases, and its structure was identified by ¹H NMR spectroscopy as an extended Le^y (lactodifucooctaosylceramide, $V^3FucVI^2FucnLc_6$; Structure 1 below). (iii) The third, major component, common in all eight cases, has been identified as trifucosyl Le^y (lactotrifucononaosylceramide, $III^3FucV^3FucVI^2FucnLc_6$, Structure 2 below) based on ¹H NMR spectroscopy, methylation analysis, and direct-probe electron-impact mass spectrometry.

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Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
  2        3
  ↑        ↑
Fucα1    Fucα1
```

STRUCTURE 1

```
Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1Cer
  2        3                 3
  ↑        ↑                 ↑
Fucα1    Fucα1             Fucα1
```

STRUCTURE 2

A large number of monoclonal antibodies directed to human cancer tissues and cell lines derived therefrom have been characterized as being directed to a series of glycolipids with Le^x, its sialylated derivative (sialyl Le^x) (for a review, see Ref. 1), and Le^y determinant (2–4).[1] Extensive studies have been performed on the isolation and characterization of a series of glycolipids having Le^x determinant, which were characterized as lactofucopentaosyl(III)ceramide (5) and mono-, di-, and trifucosyl type 2 chain ($V^3FucnLc_6$, $V^3III^3Fuc_2nLc_6$, and $VII^3V^3III^3Fuc_3nLc_8$)[2] (6, 7). Monoclonal antibodies that are capable of distinguishing these Le^x structures have been established (8). One such antibody, FH4, which reacts with dimeric Le^x, but not with monomeric Le^x, showed an immunohistological staining pattern typical for an oncofetal antigen in gastrointestinal tumors (9). However, the chemical nature of the glycolipids having Le^y determinant has not been extensively studied. Previously, lactodifucohexaosylceramide (Le^y hexaosylceramide, $III^3FucIV^2FucnLc_4$) was isolated and characterized from a liver metastatic lesion from colonic cancer and from the gastric cancer cell line MKN74 (4). A series of other glycolipids having Le^y determinant defined by the antibody AH6 have been detected in the extracts of

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This paper is dedicated to Professor Luis L. Leloir on the occasion of his 80th birthday.

‡ Supported by and on leave from the Japan Immunoresearch Laboratory, 17-5 Sakae, Takasaki, Gumma 370 Japan (present address).

[1] The nomenclature Le^x and Le^y is adopted for the structures previously designated X and Y (34), respectively, based on the fact that they are positional isomers of blood group Lewis antigens Le^a and Le^b. However, Le^x and Le^y have been identified as being unrelated to Lewis blood group antigens, *i.e.* these structures do not represent a subtype of Lewis antigens that have been designated by the suffix a, b, c, etc. (35, 36). The unknown antigen present in Le(a+b−) and Le(a−b+) erythrocytes but absent in Le(a−b−) erythrocytes was once called Le^x (37), but the antibody recognizing this structure is clearly directed to a part of the Le^a structure (38), which is unrelated to Le^x or Le^y as defined in this paper.

[2] Glycolipids are designated according to the recommendations of the Nomenclature Committee of the International Union of Pure and Applied Sciences (39); however, the suffix OseCer is omitted for shorthand designation.

various tumors and in normal blood cell membranes (4), although their chemical structures have not been elucidated. Karlsson and Larsson (10) found a glycolipid fraction ("fraction 30") in meconium consisting of hepta- to decaosylceramides containing 0–3 fucose residues. Mass spectrometry of the permethylated glycolipid mixture suggested the presence of Le^y or Le^b hepta- to decaosylceramides, although none of these components were isolated or unequivocally characterized. This paper describes the pattern of Le^y-active glycolipids defined by antibody AH6 in various cases of colonic adenocarcinoma as compared with normal tissues and blood cells. One of the major Le^y-active components, present in human colonic adenocarcinomas but absent in normal colonic extract and in normal blood cell membranes, has now been isolated and characterized as an Le^y trifucosylnonaosyl structure. This paper describes the isolation and characterization of this novel glycolipid.

MATERIALS AND METHODS[3]

RESULTS

Le^y-active Glycolipids from Human Colonic Adenocarcinoma—The polar neutral glycolipids from eight cases of human adenocarcinomas and their immunostaining pattern by anti-Le^y antibody AH6 are shown in Fig. 1, *A* and *B*. There were four groups of AH6-reactive components. (i) The fast migrating component reacting with AH6 antibody (band b) had a thin-layer chromatography mobility slightly slower than Le^x ceramide pentasaccharide ($III^3FucnLc_4$) (band a) and a slightly faster mobility than H_2 glycolipid. This component was identified previously as a lactodifucohexaosylceramide ($III^3FucIV^2FucnLc_4$) (4), which has a carbohydrate structure identical to the Le^y glycolipid of dog intestine (26). These two components overlap considerably depending on the ceramide composition. Band b as revealed by orcinol-sulfuric acid reaction was one of the major components in tumors 118 (Fig. 1*A*, *lane 1*), 845 (Fig. 1*A*, *lane 7*), and 067 (Fig. 1*A*, *lane 5*), but was a very minor component and undetectable by orcinol-sulfuric acid reaction in tumors 038 (Fig. 1*A*, *lane 3*) and 126 (Fig. 1*A*, *lane 8*). The band b component was detectable by AH6 antibody, and the intensity of immunostaining did not correspond to the chemical quantity of the band detected by orcinol-sulfuric acid. (ii) The second Le^y-active component defined by AH6 had a slightly slower TLC mobility than y_2 glycolipid ($V^3FucnLc_6$) and was found in very small chemical quantity as indicated by orcinol-sulfuric acid reaction (Fig. 1*A*, *band c*). Despite its small chemical quantity, it stained well on HPTLC[4] by antibody AH6 (Fig. 1*B*, *band c*). This minor component has been isolated from fraction VI (HPLC tubes 70–79) of tumor 845 and further purified as an acetate on HPTLC as described under "Materials and Methods" (see Miniprint Supplement). This component was characterized by ^1H NMR as Le^y-active octaosylceramide, *i.e.* difucosyllactonorhexaosylceramide ($V^3FucVI^2FucnLc_6$) as described in the following section. (iii) The third component, the slower mi-

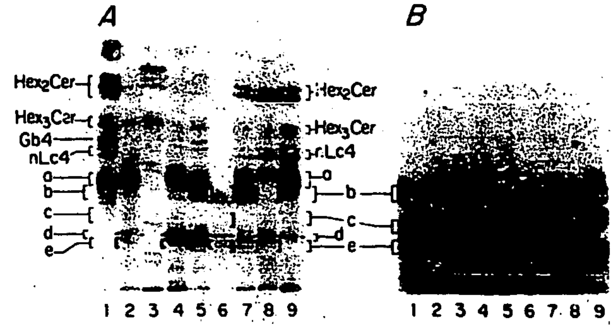

FIG. 1. **Pattern of upper neutral glycolipids of eight cases of colonic adenocarcinomas and their immunostaining with anti-Le^y monoclonal antibody AH6.** The upper neutral glycolipids of various colonic adenocarcinoma cases detected by 0.5% orcinol in 2 M sulfuric acid in *A* and immunostained with antibody AH6 in *B* are shown. *Lane 1*, TG118 (colonic adenocarcinoma metastatic to liver; host blood group AB); *lane 2*, TG105 (cecum adenocarcinoma metastatic to liver; host blood group B); *lane 3*, TG038 (sigmoid adenocarcinoma, host blood group unidentified); *lane 4*, FT75-620 (colonic adenocarcinoma metastatic to liver, host blood group O); *lane 5*, TG067 (colonic adenocarcinoma metastatic to liver; host blood group unidentified); *lane 6*, standard Le^y-active glycolipids (from *top* to *bottom*, $II^3FucIV^2FucnLc_4$, $V^3FucVI^2FucnLc_6$, and III^3FucV^3-$FucVI^2FucnLc_6$, respectively); *lane 7*, FT75-845 (colonic adenocarcinoma metastatic to liver; host blood group A); *lane 8*, TG126 (colonic adenocarcinoma metastatic to liver; host blood group O); *lane 9*, TG115 (primary liver adenocarcinoma; host blood group O). Faster migrating bands are identified on the margins: *Hex₂Cer*, ceramide dihexoside; *Hex₃Cer*, ceramide trihexoside; *Gb4* for globoside; *nLc4* for lactoneotetraosylceramide (paragloboside). Slower migrating *bands a–e* have been identified as Le^x pentaosylceramide (III^3-$FucnLc_4$), Le^y hexaosylceramide ($III^3FucIV^2FucnLc_4$), Le^y octaosylceramide (extended Le^y, $V^3FucVI^2FucnLc_6$), Le^x octaosylceramide (difucosyl y_2, $III^3V^3Fuc_2nLc_6$), and Le^y trifucosylnonaosylceramide ($III^3FucV^3FucVI^2FucnLc_6$), respectively. Note that TG038 (*lane 3*) and TG126 (*lane 8*) had very small quantities of Le^y hexaosylceramide. The intensities of immunostained bands by AH6 as shown in *B* do not match the intensities of the orcinol-sulfuric acid reaction. *Bands b, c,* and *e* correspond to three groups of Le^y antigen as described in the text.

grating glycolipid partially overlapping with dimeric Le^x ($III^3V^3Fuc_2nLc_6$) (Fig. 1*A*, *band e*), was found to be strongly stained by AH6 in all tumor cases (Fig. 1*B*, *band e*). This third Le^y component had an average TLC mobility nearly identical to H_3 glycolipid of O erythrocytes and partially overlapping with Le^x-active difucosyllactonorhexaosylceramide (dimeric Le^x, $III^3V^3Fuc_2nLc_6$), which migrated partially faster on HPLC (Fig. 1*A*, *band d*). Since dimeric Le^x showed a great deal of heterogeneity due to the difference in ceramide composition (separated in four bands) (7), a slower migrating dimeric Le^x overlaps with Le^y-active band e component. The band e component was found mainly in fraction VII (HPLC tubes 79–89; see Fig. 1 in the Miniprint Supplement) and was further purified as an acetate on HPTLC as described under "Materials and Methods" (see Miniprint Supplement). The Le^y-active component was characterized as a nonaosylceramide (trifucosyllactonorhexaosylceramide, III^3FucV^3Fuc-$VI^2FucnLc_6$) as described in the following section. (iv) The slower migrating unidentified components that were chemically very minor were stained strongly by AH6 antibody.

^1H NMR Spectrum of Le^y-active Octaosylceramide (Band c) and Nonaosylceramide (Band e)—The anomeric region of the ^1H NMR spectrum of the band c component, a minor Le^y-active component of adenocarcinoma prepared from FT 620 tumor, is reproduced in Fig. 2*A*. Comparison with the spectra of other known type 2 chain glycolipids obtained under the same conditions (27) showed clearly that this spectrum differs from that of the Le^y-active hexaglycosylceramide

[3] Portions of this paper (including "Materials and Methods" and additional Figs. 1–4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-341, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[4] The abbreviations used are: HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography; HexNAc, *N*-acetylhexosamine.

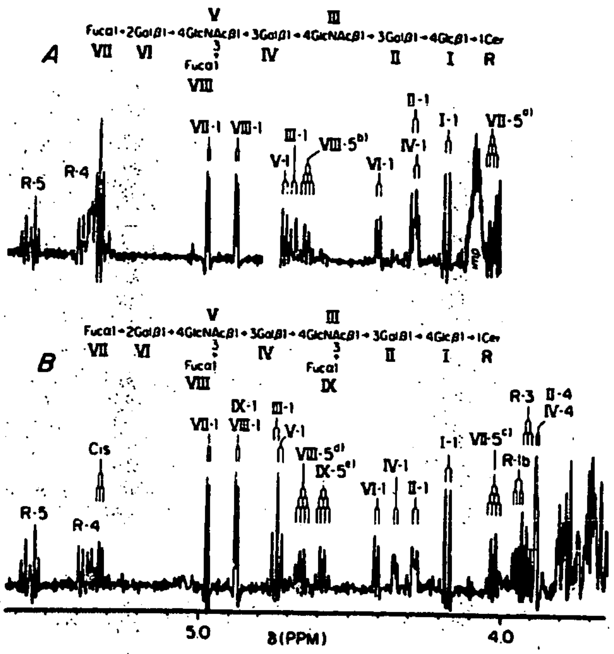

FIG. 2. **Downfield regions of resolution-enhanced 500-MHz ^{1}H NMR spectra of adenocarcinoma glycolipids from tumor 620 V^{3}FucVI^{2}FucnLc$_6$ (*A*) and III^{3}FucV^{3}FucVI^{2}FucnLc$_6$ (*B*) at 328 K, (concentration of 0.1–0.2 mM).** 3640 and 3800 free induction decays were accumulated for *A* and *B*, respectively, and resolution was enhanced by application of a gaussian window function prior to transformation. An impurity peak at 4.77 ppm in *A* was deleted. The assignments of II-1 and IV-1 in *A* may be reversed; *a–e*, Fuc H-5 resonances confirmed by decoupling from CH_3 of same residue. *Arabic numerals* refer to ring protons of residues designated by *roman numerals* in the corresponding structure.

(III^{3}FucIV^{2}FucnLc$_4$) (4) solely in the addition of two anomeric signals from non-fucosylated internal β-GlcNAc and β-Gal residues. Thus, the anomeric resonances at 4.973 ppm ($^3J_{1,2}$ = 3.7 Hz), 4.404 ppm ($^3J_{1,2}$ = 6.7 Hz), 4.875 ppm ($^3J_{1,2}$ = 3.7 Hz), and 4.712 ppm ($^3J_{1,2}$ = 7.9 Hz), along with the Fuc H-5 quartets at 4.027 and 4.640 ppm coupled to Fuc CH_3 doublets (not shown) at 1.103 and 1.072 ppm ($^3J_{5,6}$ = 6.7 Hz), are fully diagnostic for the Le^y-terminal tetrasaccharide. These resonances are assigned as shown in Fig. 2*A*. The four remaining anomeric resonances at 4.285 ppm ($^3J_{1,2}$ = 7.3 Hz), 4.684 ppm ($^3J_{1,2}$ = 7.9 Hz), 4.276 ppm ($^3J_{1,2}$ = 7.3 Hz), and 4.172 ppm ($^3J_{1,2}$ = 7.9 Hz) are consistent with the four unsubstituted internal residues of nLc$_6$, as assigned in Fig. 2*A*. In addition, 2 GlcNAc/NAc singlets were found at 1.820 ppm (6H, not shown), unshifted from the position found for nLc$_6$. Thus, the structure of this glycolipid could be identified as V^{3}FucVI^{2}FucnLc$_6$ from its ^{1}H NMR spectrum alone. The resonance shifts are all likewise consistent with substitution of the H_2 glycolipid by Fucα1→3 at GlcNAc V or with y_2 glycolipid substituted by Fucα1→2 at Gal VI (27, 28).

The anomeric region of the ^{1}H NMR spectrum of the major Le^y-active band e component from FT 620 tumor is reproduced in Fig. 2*B*. Using the data for V^{3}FucVI^{2}FucnLc$_6$ as a base and the knowledge of glycosylation-induced shift effects for type 2 chain structures (28), the structure of this glycolipid could also be deduced from its ^{1}H NMR spectra. Thus, the presence of the additional Fucα1→3 at GlcNAc III was evident not only by the H-1, H-5, and CH_3 resonances for this unit (4.876 ppm ($^3J_{1,2}$ = 4.3 Hz),[5] 4.586 ppm, and 1.014 ppm ($^3J_{5,6}$ = 6.7 ppm), respectively), but also by its predictable effect on H-1 of GlcNAc III (Δδ = 0.059 ppm), Gal IV (Δδ = 0.067 ppm), and GlcNAc V (Δδ = 0.014 ppm) and H-5 of Fucα1→3GlcNAc V (Δδ = 0.013 ppm). The shifts are equally in accord with the conversion of III^{3}FucV^{3}FucnLc$_6$ by addition of a terminal Fucα1→2 unit. In addition, the line shapes for each of the Gal H-1 resonances are consistent with the virtual coupling phenomenon detected in the simpler analogs. A detailed discussion of the ^{1}H NMR spectra of type 2 chain-based Le^x and Le^y structures will be presented elsewhere (28). The assigned oligosaccharide structure is consistent with its antibody reactivity and HPTLC behavior and was confirmed subsequently by direct-probe electron-impact mass spectrometry of the permethylated compound and gas chromatography-mass spectrometry of its hydrolysis products (see below).

In addition to information regarding oligosaccharide structure, ^{1}H NMR also gave some indication of ceramide composition. Thus, the spectrum of trifucosyl Le^y from tumor 620 (Fig. 2*B*) showed sphingosine *trans*-vinyl proton signals at 5.37 ppm (R-4) and 5.45 ppm (R-5), R-1b at 3.94 ppm (3.98 ppm at 308 K), and a small *cis*-vinyl triplet at 5.32 ppm representing approximately 20–30% unsaturated fatty acids. This, along with the single β-Glc H-1 signal at 4.171 ppm, indicates a ceramide composition similar to that found, for example, in normal human erythrocytes (29). The spectrum of trifucosyl Le^y obtained from the tumor 845 (see Fig. 4 in the Miniprint Supplement) was identical in the anomeric region in every respect except that the β-Glc H-1 signal was found primarily at 4.222 ppm. This was accompanied by a virtual absence of sphingosine *trans*-vinyl signals, an approximately 4-fold elevation in the level of the fatty acid *cis*-vinyl proton signal, and the appearance of R-1b of sphingosine predominantly at 4.05 ppm at 308 K. These features have previously been identified with a ceramide of G_{M_4} from mullet roe containing phytosphingosine and a high proportion of α-hydroxylated, unsaturated fatty acids (30). Similar features are also apparent in the spectra of poly-Le^x fucolipids previously isolated from adenocarcinoma and shown to have a high content of α-hydroxy fatty acids (7). This sample was permethylated for the subsequent studies by gas chromatography-mass spectrometry and direct probe mass spectrometry.

Methylation Analysis of the Le^y-active Nonaosylceramide—The structure of the major Le^y-active nonaosylceramide as III^{3}FucV^{3}FucVI^{2}FucnLc$_6$ as suggested by ^{1}H NMR above has been fully confirmed by methylation analysis. Gas chromatography-mass spectrometry (Fig. 3) of the partially methylated alditol acetates produced upon hydrolysis of permethylated Le^y-active nonaosylceramide from FT 845 tumor yielded alditol acetates of 2,3,4-tri-*O*-MeFuc, 2,3,6-tri-*O*-MeGlc, 2,4,6-tri-*O*-MeGal, 3,4,6-tri-*O*-MeGal, and 6-mono-*O*-Me-GlcNAcMe on a DB-5 column (Fig. 3*A*). The identity of the peak containing the two tri-*O*-MeGal derivatives was confirmed by chromatography on a DB-225 column (Fig. 3*C*), whereas the 6-*O*-MeGlcNAcMe was confirmed by coinjection with an authentic sample. For comparison, the gas chromatography-mass spectrometry of partially methylated alditol acetates obtained from III^{3}FucV^{3}FucnLc$_6$ of the same tumor is reproduced in Fig. 3, *B* and *D*, which shows that the only qualitative difference is the elimination of the terminal Gal derivative (2,3,4,6-tetra-*O*-MeGal) and the concomitant appearance of 3,4,6-tri-*O*-MeGal in the top chromatograms.

Direct-probe Electron-impact Mass Spectrometry of Le^y-

[5] The appearance of this resonance as being equal in amplitude to the Fuc H-1 at 4.973 ppm is a distortion resulting from resolution enhancement. Integration of the unenhanced spectrum shows that this resonance contains two protons.

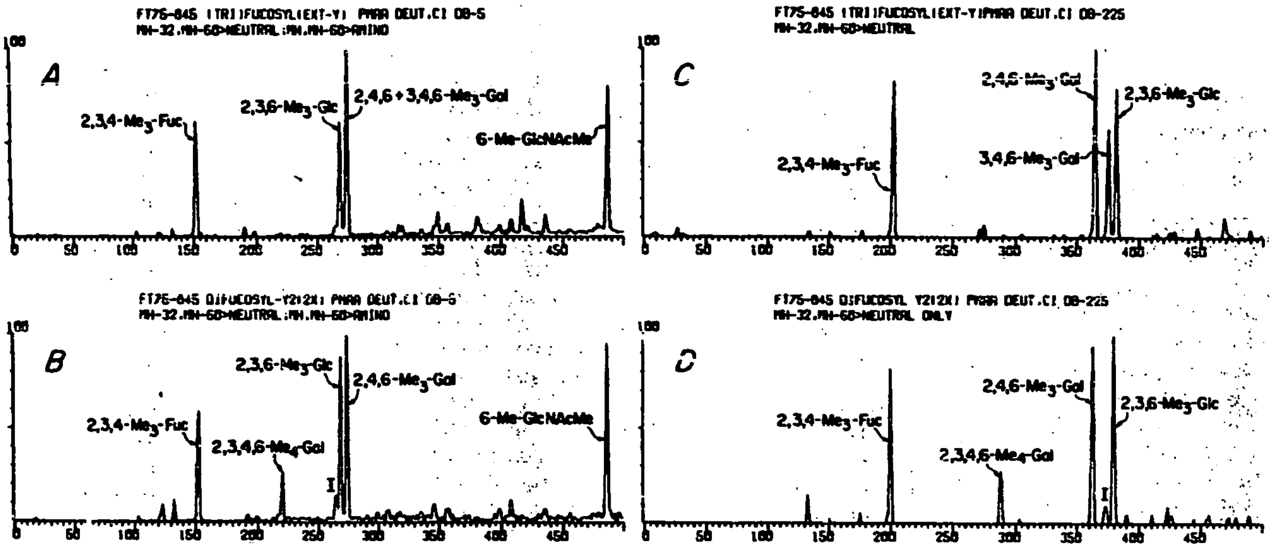

FIG. 3. **Partially *O*-methylated alditol and hexosaminitol acetates detected in the hydrolysate of permethylated glycolipids.** *A*, partially *O*-methylated alditol acetates of III^{3}FucV^{3}FucVI^{2}FucnLc$_6$ separated on a DB-5 column; *B*, for comparison, partially *O*-methylated alditol acetates of III^{3}FucV^{3}FucnLc$_6$ (7) chromatographed under identical conditions on a DB-5 column; *Panel C*, partially *O*-methylated alditol acetates of III^{3}FucV^{3}FucVI^{2}FucnLc$_6$ chromatographed on a DB-225 column to show separation of 2,4,6- from 3,4,6-tri-*O*-MeGal; *D*, partially *O*-methylated alditol acetates of III^{3}FucV^{3}FucnLc$_6$ chromatographed on a DB-225 column under identical conditions. *Ordinate*, sum of ion intensities of MH^+, $(MH-60)^+$ for *N*-acetylhexosaminitols and $(MH-32)^+$, $(MH-60)^+$ for neutral alditol derivatives; *abscissa*, scan number. Detailed conditions are as described previously (20).

active Nonaosylceramide—The structural assignment predicted by ^{1}H NMR spectroscopy and methylation analysis was further confirmed by direct-probe electron-impact mass spectrometry of the permethylated FT 845 tumor glycolipid. The spectrum and fragmentation pattern are shown in Fig. 4, *A* and *B*, respectively. The most prominent carbohydrate ions are derived, as expected, from cleavages at HexNAc, producing the difucosyltetrasaccharide series *m/z* 812→780 and the trifucosylheptasaccharide series *m/z* 1435→1403. Preferential loss of the neutral 3-substituent from HexNAc (11, 31) of the primary fragments produces the ions *m/z* 606 and 1229, respectively. This is consistent with the proposed type 2 chain structure since the ion *m/z* 402, which would be produced if the Hex-containing fragment were linked to the 3-position of HexNAc, is not prominent. The prominent ion *m/z* 182 is similarly an indication of type 2 chain structure. The carbohydrate structure is further confirmed by ions of lower abundance at *m/z* 189→157 (deoxy-Hex-), 361 (from 393, deoxy-Hex-*O*-Hex-), and 1016→984 (difucosylpentaose fragment). Ceramide composition is indicated by the ions shown in Fig. 4*B*. In particular, the A series (31) appears characteristic of ceramides containing phytosphingosine, which is also represented by the ion at *m/z* 396. Virtually no ion for unsaturated sphingosine species is detectable at *m/z* 364, in agreement with the ^{1}H NMR. The ambiguity remaining due to the duplication of ions of different fatty acid composition (*i.e.* 22:1 α-OH and 24:0 fatty acids produce fragments of the same mass) would best be settled by fatty acid analysis, but this was not possible since no more of this sample was available. ^{1}H NMR does indicate a high proportion of unsaturated α-OH fatty acids in combination with phytosphingosine, as mentioned earlier. In the mass spectrum, the series of ions produced by loss of oxygen (−16) from the A fragments is prominent. Previously, these have been seen in the electron-impact mass spectra of H type 1 chain fucolipid isolated from human plasma (31), Le^b fucolipid from dog intestine (32), and fucolipids isolated from human meconium (10), attributable in each case to ceramides containing phytosphingosine and saturated α-hydroxy fatty acids. In the present case, there is a predominance of ions 2 atomic mass units less than those reported, indicating unsaturated α-OH fatty acids, consistent with ^{1}H NMR analysis. Ions attributable to ceramide plus internal tetrasaccharide are found in the higher mass range at *m/z* 1491, 1517, and 1521.

DISCUSSION

One of the characteristic features of carbohydrate changes in human cancer, particularly in colonic adenocarcinoma, is the accumulation of glycolipids having Le^y determinant, which is defined by various monoclonal antibodies prepared by independent laboratories (2–4). Although a small quantity of Le^y antigen is widely distributed in some normal epithelial tissues, glandular tissues, and their secretions, it is essentially absent in the distal region of normal colonic epithelia, including the sigmoidal colon and rectum, except in the crypt areas (33). A large quantity of the antigen is present, however, in essentially all adenocarcinomas of colon, irrespective of their location, and in the majority of colonic adenomas and polyps that have potential malignant properties (such as villous and villotubular adenomas), but is absent or weakly expressed in nonmalignant adenomas and polyps, such as tubular and juvenile polyps (33). Thus, the expression of Le^y antigen in colonic cancer and polyps has diagnostic and prognostic value. In a previous study, we characterized the fast migrating, simplest Le^y-active glycolipid as lactodifucohexaosylceramide (4). A significant quantity of this glycolipid was found in six out of eight cases of colonic adenocarcinomas, although two cases (tumors 126 and 038) of colonic adenocarcinoma contained only a very small quantity, as shown in Fig. 1 (see *lanes 3* and *8*). In all cases so far examined, a consistent major component was found to be a slow migrating Le^y-active glycolipid, which is described in this paper. This glycolipid was

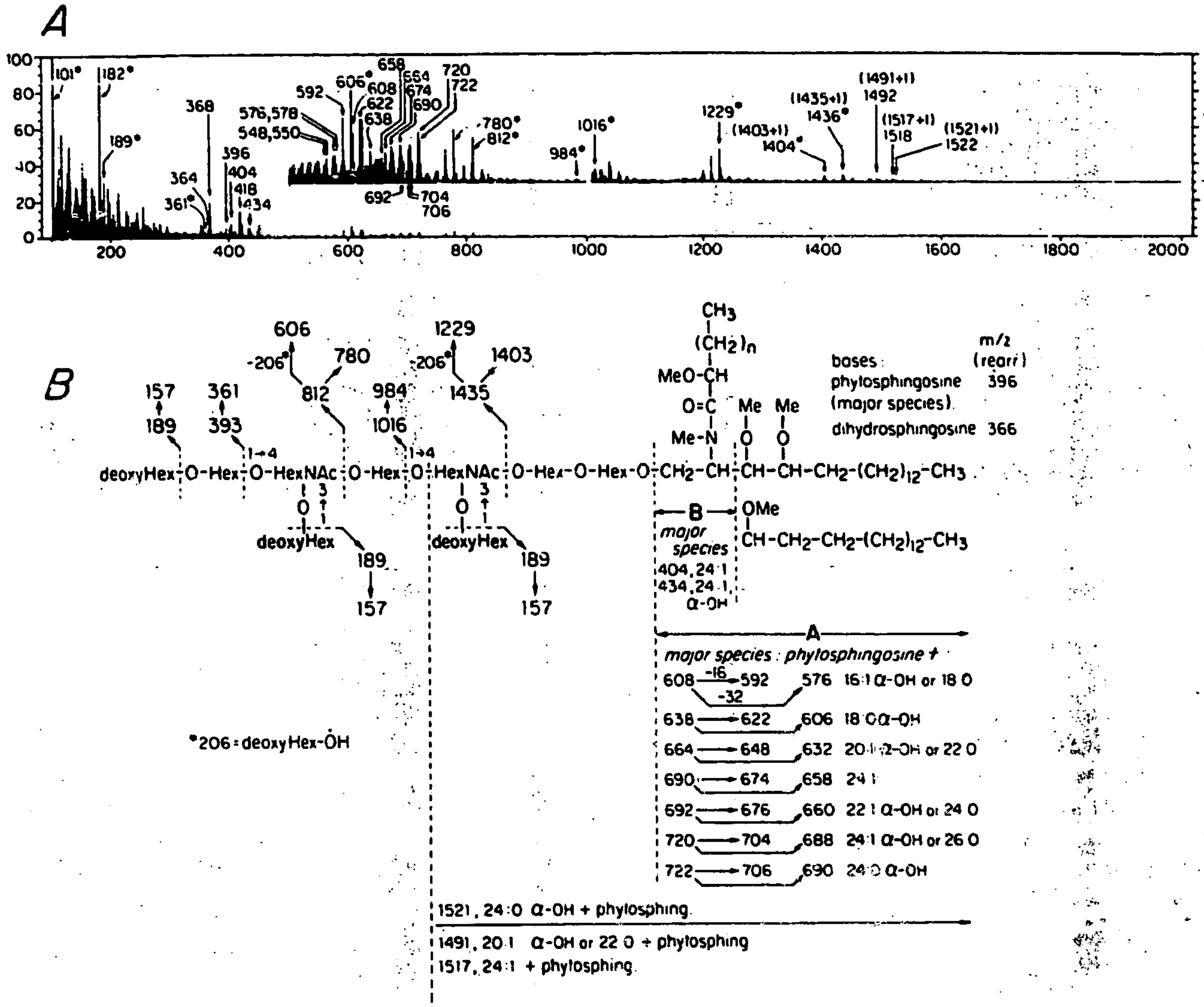

FIG. 4. *A*, electron-impact mass spectrum of permethylated III³FucV³FucVI²FucnLc₆ from 845 adenocarcinoma. Ions marked by *asterisks* are attributed mainly to nonreducing end carbohydrate ions. *B*, fragmentation pattern producing the characteristic ions indicated in the mass spectrum.

identified by ^{1}H NMR spectroscopy, methylation analysis, and direct-probe mass spectrometry as trifucosyl Le^y (Le^y nonaosylceramide, III^{3}FucV^{3}FucVI^{2}FucnLc$_6$), as described under "Results." This structure is the product of the addition of 1 fucosyl residue to the terminal Gal residue of Le^x-active difucosyloctaosylceramide (III^{3}FucV^{3}FucnLc$_6$, difucosyl y$_2$, dimeric Le^x), which is the major Le^x-active component of human adenocarcinoma (7). A minor component, which had a slightly slower TLC mobility than H$_2$ glycolipid, was isolated and characterized by ^{1}H NMR spectroscopy as difucooctaosylceramide (V^{3}FucVI^{2}FucnLc$_6$).

The presence in human meconium of a series of hepta- to decaosylceramides having Le^a, Le^y, and Le^b determinants with or without internal fucosylation was suggested by total mass spectrometry of a permethylated mixture (10). Although these components were not isolated or characterized (10), the abundance of polyfucosylated glycolipids in both meconium and human colonic cancer may indicate that the expression of these structures is oncodevelopmentally regulated. The internal $\alpha1\rightarrow3$ fucosyl substitution of the major Le^y nonaosylceramide provides an interesting antigenicity for this glycolipid, which will be described in the accompanying paper (40).

Acknowledgments—We thank Drs. Catherine Costello and Klaus Biemann of the Massachusetts Institute of Technology and Tetsuo Higuchi of JEOL for analysis of glycolipids by direct-probe mass spectrometry using their JEOL HX110 equipment.

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SUPPLEMENTAL MATERIAL FOR
NOVEL FUCOLIPIDS OF HUMAN ADENOCARCINOMA. V.
CHARACTERIZATION OF THE MAJOR Le^y ANTIGEN OF HUMAN ADENOCARCINOMA
AS TRIFUCOSYL NONASYL Le^y GLYCOLIPID (III^3FucV^3FucVI^2FucnLc$_6$)

By Edward Nudelman, Steven B. Levery, Tokio Kaizu, and Sen-itiroh Hakomori

MATERIALS AND METHODS

Materials.

Various human cancer tissues were obtained through the courtesy of Ms. Wilma Verrato of the Tumor Procurement Program of the National Institutes of Health. Colonic adenocarcinoma metastatic deposits in liver, PT75-620, PT75-845, and TG118 were used in this study, since these tumors contained a relatively large quantity of glycolipids having Le^y trifucosyl-nonaosylceramide. Le^y octaosylceramide was isolated from tumor TG118. Lactodifucohexaosyl-ceramide (III Fuc IV Fuc nLc$_4$) was isolated from tumor PT75-845. Five other colonic adenocarcinoma cases as described in the legend of Fig. 1 were analysed for glycolipid patterns and their immunostaining pattern with anti-Le^y antibody AH6.

Extraction and fractionation of glycolipids.

Extraction and preparation of the upper neutral glycolipid fraction: Tissues were extracted three times with 5 volumes (w/v) of isopropyl alcohol-hexane-water (55:25:20, v/v/v) (11), and the extracts were evaporated to dryness. The residue was dissolved in 5 volumes to the original tissue wet weight (w/v) of chloroform-methanol (2:1, v/v) and solvent-partitioned after addition of one-sixth volume of water by a modified Folch's method (12). The upper phase glycolipid fraction was separated by four times repeated solvent partition, and the fraction was evaporated in a rotary evaporator to a small volume (20-30 ml) and dialysed through a Spectrapor 3 dialysis tube (Spectrum Medical Industries, Los Angeles, CA) (12) followed by DEAE-Sephadex chromatography in chloroform-methanol-water (30:60:8, v/v/v) (13). The upper neutral glycolipid fraction was evaporated to dryness. The average yield of the upper neutral glycolipids was approximately 30–40 mg from 100 g of wet tissue.

Fractionation by high performance liquid chromatography (HPLC): The fraction was further separated into components on HPLC employing a 1 x 50 cm column packed with Iatrobeads 6RS-8010, pre-equilibrated with isopropyl alcohol-hexane-water (55:40:5, v/v/v) according to the method described previously by Watanabe and Arao (14) and modified by Kannagi et al. (10,15). The glycolipid fraction (50-100 mg) dissolved in 2-3 ml of chloroform-methanol-water (60:35:5, v/v/v) was injected onto the Iatrobeads column and eluted with a solvent gradient of isopropyl alcohol-hexane-water from 55:40:5 to 55:25:20 (v/v/v) during 150 min and further eluted with the same solvent for an additional 50 min. Pressure was applied to obtain a flow rate of 1.0 ml/min, and the eluate was collected on a fraction collector at 2.0 ml/tube. A 5 µl aliquot of each fraction was analysed on HPTLC (Merck, Darmstadt, West Germany) and developed in a solvent mixture of chloroform-methanol-water (50:40:10, v/v/v), and spots were revealed with 0.5% orcinol in 2 N sulfuric acid. The separation pattern of glycolipids is shown in Fig. 1 of the Supplemental Material. Tubes 55–60 (fraction IV) contained Le^x ceramide pentasaccharide partially overlapping with Le^y ceramide hexasaccharide; tubes 61–69 (fraction V) contained mainly Le^y ceramide hexasaccharide and a minor Le^x-active ceramide (V^3Fuc nLc$_6$); tubes 70–79 (fraction VI) contained Le^y-active ceramide octasaccharide, Le^y-active ceramide nonasaccharide, and Le^x-active ceramide octasaccharide (III^3 V^3 Fuc$_2$ nLc$_6$); and tubes 80–89 (fraction VII) contained mainly Le^y-active ceramide nonasaccharide (which was identified as trifucosyl Le^y), and minor Le^x-active ceramide octa-, nona-, and decasaccharides. These tumors have been characterized by the presence of a series of both Le^x and Le^y glycolipids having ceramide compositions with a large degree of variation (7). Le^x glycolipids with short chain fatty acids or hydroxylated fatty acids or sphingosines overlap with Le^y glycolipids with long chain fatty acids and non-hydroxylated ceramide species. Therefore, the major difficulty in isolating Le^y-active components is their separation from Le^x-active components. This was performed by repeating the HPLC separation and HPTLC of acetylated derivatives as described below, monitored with ¹H-NMR spectroscopy, through which glycolipids with Le^y determinant can be distinguished from those with Le^x determinant quantitatively.

Further purification by second HPLC and HPTLC as acetates: The fraction that contained eluates in tubes 80–89 above was further separated by a second HPLC through a smaller column (1 x 30 cm) of Iatrobeads 6RS-8010 eluted with a gradient of isopropyl alcohol-hexane-water from 55:40:5 to 55:25:20 (v/v/v) during 60 min, followed by elution with the same solvent for an additional 20 min. Pressure was applied to obtain a flow rate of 0.5 ml/min, and the eluate was collected on a fraction collector at 0.5 ml/tube. Tubes 42–47 were pooled and termed fraction A; tubes 48–52 were pooled and termed fraction B. Glycolipids in these fractions were further separated, after acetylation, on HPTLC plate (Merck) developed with dichloroethane-acetone-water (50:50:0.1, v/v/v). Separation was improved by developing the plate twice with intermediate drying. Bands were revealed by Primulin spray (Aldrich Chemical Co, Milwaukee, WI) and were observed under ultraviolet light (16). Glycolipid acetate bands were scraped from the plate and extracted in chloroform-methanol (2:1, v/v) by sonication followed by centrifugation. Seven bands were separated from fraction A (termed A$_1$-A$_7$) and three bands were separated from fraction B (termed B$_1$-B$_3$). These acetate bands separated after HPTLC, are shown in Fig. 2 of the Supplemental Material. After deacetylation in chloroform-methanol (2:1, v/v) containing 1% sodium methoxide, each fraction was analysed by ¹H-nuclear magnetic resonance spectroscopy in a 500 MHz Bruker spectrometer by the method described below. The NMR spectra of glycolipids present in each fraction, separated as acetates, indicated that A$_1$, A$_2$, and A$_4$ were essentially (>90%) trifucosyl Le^y (differences may be due to ceramide composition). These fractions were pooled and further purified by preparative HPTLC, and essentially pure Le^y glycolipid was isolated from this fraction. This isolated Le^y was used for structural analysis as well as for antibody preparation. The HPTLC pattern of the purified Le^y nonaosylceramide and dimeric Le^x are shown in Fig. 3 of the Supplemental Material. A$_3$ contained about 60% Le^y and 40% Le^x. A$_5$ and A$_6$ contained 90% Le^x and A$_7$ contained 100% Le^x. The Le^x component in these fractions represents mainly dimeric Le^x (III^3 V^3 Fuc$_2$ nLc$_6$). B$_1$, B$_2$, and B$_3$ contained 50% Le^x and 50% Le^y, although these components showed a single band on HPTLC.

Determination of antigen activity

Immunostaining of glycolipids separated on HPTLC (Baker Si-HPF TLC Plate, J.T. Baker Chemical Co., Phillipsburg, N.J.) was performed by a modification (10,17) of the method originally described by Magnani et al. (18). The monoclonal antibody AH6 directed to Le^y determinant (Fucα1→2Galβ1→4[Fucα1→3]GlcNAcβ1→3Galβ1→R) was prepared as previously described by Abe et al. (4).

Chemical characterization of glycolipids

Methylation analysis: The purified glycolipids (100 µg) were permethylated (19) and subjected to hydrolysis, reduction, and acetylation and were analysed by gas chromatography/methane chemical ionization mass spectrometry as previously described (20-22).

Direct probe electron-impact mass spectrometry of permethylated glycolipids: The permethylated glycolipids were analysed by direct probe mass spectrometry (23,24) in electron-impact mode using a high mass range mass spectrometer (JEOL HX110). Each sample (approximately 5 µg) dissolved in 3 µl of ethanol was placed in a direct probe cuvette, and the spectrum was obtained with ionization potential 7 eV, ionizing current 300 µA, accelerating voltage 10 KV. The temperature was raised from 200°C to 380°C, and significant spectra appeared at 240–280°C. Modified direct insertion probe was used.

¹H-NMR spectroscopy: For ¹H-NMR, 100–150 µg of deuterium-exchanged purified glycolipids were dissolved in 0.4 ml of dimethylsulfoxide-d$_6$ containing 2% D$_2$O (25) and 1% tetramethylsilane. One-dimensional spectra were obtained on a Bruker WM-500 spectrometer equipped with an Aspect 2000 computer, using quadrature detection and a 90° pulse angle; sweep width was 5 KHz collected over a 16K data set (digital resolution = 0.6 Hz/pt), giving an acquisition time of 1.684 sec, to which was added a 2 sec delay. Further details are given in the legend to Fig. 2.

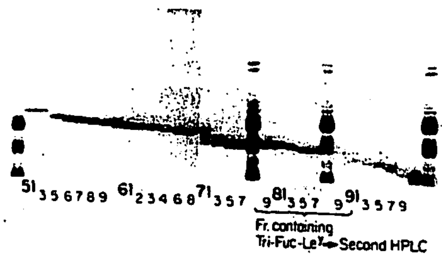

Fig. 1. The separation pattern of upper neutral glycolipids of colonic tumor 845 on HPLC. The HPLC program is described in the text. The glycolipid pattern of tubes 51-100 separated by HPTLC in chloroform-methanol-water (50:40:10, v/v/v) is shown in this figure. Tubes 51-53 are nLc$_4$; tubes 55-60 are mainly III^{3}FucnLc$_4$; tubes 61-69 are III^{3}FucIV^{2}FucnLc$_4$; tubes 70-74 are III^{3}FucV^{3}FucnLc$_6$ and V^{3}FucVI^{2}FucnLc$_6$ having various types of ceramides; tubes 80-89 are III^{3}FucV^{3}FucVI^{2}FucnLc$_6$. Tubes 80-89 were combined and further purified by a second HPLC as described in the text.

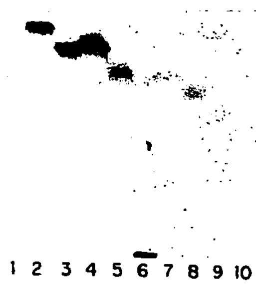

Fig. 2. HPTLC separation pattern of glycolipid acetates having Le^x and Le^y octa- and decasaccharides. Acetates of fractions A and B, which were obtained from the second HPLC on an Iatrobeads 6RS-8010 column, were separated on HPTLC as described under the Materials and Methods. Lanes 1-7 are fractions A$_1$ to A$_7$, respectively, which were separated from fraction A. Lanes 8-10 are fractions B$_1$ to B$_3$, respectively. Bands 1 and 2; 6 and 7; and 9 and 10 were not separated in the above HPTLC figure since this plate was developed only once. If the chromatogram were developed twice with intermediate drying, these bands would be separated. Band 1 was the faster-migrating portion and band 2 was the slower-migrating portion of a pair of bands running close together; a similar situation exists for bands 6 and 7 and bands 9 and 10. These bands, after deacetylation, were not separated under regular HPTLC conditions. NMR spectrometry of these bands indicated that A$_1$, A$_2$, and A$_4$ were essentially trifucosyl Le^y. For the composition of other fractions, see the text.

Fig. 3. HPTLC pattern of purified trifucosyl Le^y glycolipid (III^{3}FucV^{3}FucVI^{2}FucnLc$_6$) as compared with purified dimeric Le^x. Lane 1, tumor 620; lanes 2 and 5, tumor 845; lane 3, purified dimeric Le^x; lane 4, purified Le^y trifucosylnonaosylceramide; lane 6, purified upper neutral glycolipids from O blood cells. HPTLC plate was developed in chloroform-methanol-water (50:40:10, v/v/v) and was stained by 0.5% orcinol in 2 N sulfuric acid. The position of globoside, H$_1$, H$_2$, and H$_3$ glycolipids in O blood cell membranes is indicated in the right margin and that of Le^x and Le^y antigens is indicated in the left margin. a, III^{3}FucnLc$_4$; b, III^{3}FucIV^{2}FucnLc$_4$; c, V^{3}FucVI^{2}FucnLc$_6$; d, III^{3}FucV^{3}FucnLc$_6$; e, III^{3}FucV^{3}FucVI^{2}FucnLc$_6$.

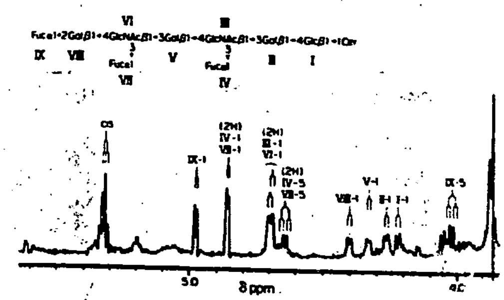

Fig. 4. Downfield region of 500 MHz ^{1}H-NMR spectrum of adenocarcinoma glycolipid III^{3}FucV^{3}FucVI^{2}FucnLc$_6$ from 845 tumor acquired at 308°K. (Note that the numbering system of residues is different from that of the previous NMR figure).